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Submitted: Photochemistry and Photobiology
January, 1992

The Effect of Triton X-100 on Purple Membrane as Measured by Changes in the Dynamics

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19960103 223

Running Title: The Effect of Triton on Dynamics

Key Words: Bacteriorhodopsin, Purple Membrane, Photocycle, Light Scattering, Membrane Dynamics, Triton X-100, Dynamics

List of Abbreviations:
bR (bacteriorhodopsin)
pm (purple membrane)
Nd:YAG (neodyminum doped yttrium aluminum garnet)
cw. (continuous wave)

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DTIC QUALITY INSPECTED 1

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget. Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (*Leave Blank*)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	See Title Page	1 February 1987 to 31 January 1991

4. TITLE AND SUBTITLE

Use Title on Reprint

5. FUNDING NUMBERS

N00014-87-C-0146
0R0A444C-
43051
43051S4

6. AUTHOR(S)

See Individual Articles

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Vanderbilt University
Nashville TN 37332
(615-322-2786)

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Office of Naval Research
800 North Quincy Street
Arlington, VA 22217-5660

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Each Paper Summarized on first page. Journal articles submitted as contract reports. All work performed under Government contract.

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (*Maximum 200 words*)

See first page of Article

14. SUBJECT TERMS

Free Electron Lasers Medicine Biology
Biomedical Instrumentation
Energy Cells

15. NUMBER OF PAGES

00

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std 239-18
298-102

Abstract:

We have observed the light scattering transients arising from changes in the curvature of purple membrane fragments upon photoexcitation at pH 8.05 and 4.1 with and without treatment of Triton X-100. The low ionic strength room temperature suspensions are excited with 532 nm light pulses from a Nd:YAG laser (20 ns). The scattering of 320 nm light is monitored from 3 μs to 1 s at scattering angles from 15° to 60°. We simultaneously measure the transient transmission changes at 320 nm. The transient light scattering signals change significantly with the addition of 0.02% Triton X-100 at pH 8.05 and 0.006% Triton X-100 at pH 4.1. At these concentrations of Triton we observe maximal amplitudes in the transient changes of the scattered light intensity. At higher concentrations, the Triton solubilizes the protein and the scattering signals are completely attenuated. The transient transmission changes become severely distorted by the scattering changes in the Triton treated samples. We can explain these changes using our bent membrane model and assuming a greater initial curvature and an increased transient curvature change in the membrane fragments after the Triton X-100 is added. The amplitudes of the scattering changes as a function of the scattering angle from 15° to 60° agree with model calculations of the scattering amplitudes.

I. Introduction

Bacteriorhodopsin (bR) is the well studied photo-activated proton pump found in the purple membrane (pm) patches of the bacteria *Halobacterium halobium* (Stoeckenius and Rowen, 1967; Oesterhelt and Stoeckenius, 1971; Stoeckenius *et al.*, 1979). After the absorption of a photon, the protein progresses through a

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sequence of intermediate states during a photocycle. These states are evidenced by changes in the optical absorption spectrum (Stoeckenius and Lozier, 1974). There have been attempts to link these absorption changes with significant rearrangements of the protein conformation. Many studies, on the contrary, measure little or no significant movement of the retinal chromophore during the photocycle (Razi Naqvi, *et al.*, 1973; Cherry, *et al.*, 1977a; Cherry, *et al.*, 1977b; Sherman and Caplan, 1977; Korenstein and Hess, 1978). Also, investigations of the protein side chains show no evidence for motion during the photocycle (Czégé, *et al.*, 1982). Time resolved x-ray diffraction also shows only a small amount of bR structure change 1 ms after illumination (Frankel and Forsyth, 1985). A one to two degree tilt of the 3 or 4 transmembrane alpha helixes during the photocycle has been measured with neutron diffraction (Dencher, *et al.*, 1989). Some motion is probably involved in the photocycle since it can be influenced by the viscosity of the surrounding medium (Beece, *et al.*, 1981). When the membrane fragments are suspended in the higher viscosity medium of glycerol/water, the photocycle is slowed. The motion of the proton moving across the membrane during the photocycle strongly suggests some protein motion. The proton motion has been directly measured by the protein electric response signal (PERS) method (Keszthelyi and Ormos, 1980; Keszthelyi, 1984).

The bR is found as trimers in the pm patches (Oesterhelt and Stoeckenius, 1973; Henderson and Unwin, 1975; Stoeckenius *et al.*., 1979). The trimers are regularly arranged in a two dimensional crystal lattice (Blaurock and Stoeckenius, 1971). The protein accounts for about 77% of the membrane by dry weight (Kushwaha, *et al.*, 1975; Stoeckenius and Bogomolni, 1982; Lozier and Parodi, 1984).

This translates to roughly 10 lipid molecules per bR molecule.

The chromophore of the protein is a retinal attached to the lysine 216 residue of the protein with a protonated Schiff base (Heyn, *et al.*., 1988). The angle of the visible absorbance dipole of the chromophore has been determined to be about 70° with respect to the membrane normal (Heyn, *et al.*, 1977; Lin and Mathies, 1989). In the light adapted state, bR has a broad absorption band centered at 568 nm (ϵ = 63,000 $cm^{-1}M^{-1}$) (Becher, *et al.*, 1978).

The pm fragments are thin sheets. Measurements have determined that they are slightly less than or approximately 0.6 μm in diameter (Barabás *et al.*, 1983; Arrio *et al.*, 1986) and approximately 4 to 5 nm thick (Blaurock, 1975; Tristram–Nagle *et al.*, 1986). Our recent work has shown that the membrane fragments change their curvature, or "flap" during the photocycle (Czégé, 1987a,b; 1988; Czégé and Reinisch, 1990; 1991a-c). The surface of the pm fragment is asymmetrically charged (Keszthelyi, 1980; Renthal and Chung, 1984; Ehrenberg and Berezin, 1984). Since the most energetically favorable configuration for a flexible membrane with an asymmetric charge distribution is curved, it is reasonable to assume that the pm fragment is curved. The charge asymmetry is pH dependent and changes sign near pH 5 (Barabás *et al.*, 1983). From the change in sign of the asymmetric charge distribution it follows that the initial curvature is reversed near pH 5.

We observe transient changes in the scattering cross section at 320 nm of the pm fragments during the photocycle. We use 320 nm because of the large scattering cross section for this wavelength. The 320 nm light does not photodestroy the bR. Also, the absorption and the transient absorption changes are minimal at 320 nm. We interpret these scattering changes to be due to changes in the curvature of the

membrane fragment.

Here, we investigate the transient scattering changes from suspensions of pm before and after treatment with Triton X-100. We use small concentrations of Triton, which do not solubilize the bR.

The detergent is expected to interact with hydrophobic parts of the protein structure. This should change the physical characteristics of the membrane sheet. Since the light scattering kinetics are sensitive to the changes in curvature, we expect to see significant changes.

Materials and Methods

Purple membrane fragments are isolated from the strain S9 of *Halobacterium halobium* according to the procedure of Oesterhelt and Stoeckenius, 1974. The samples are graciously provided by Dr. J.K. Lanyi and Dr. György Váró. The samples are suspended in 10 mM sodium acetate buffer for the pH 4.1 and in 10 mM potassium dihydrogen phosphate at pH 8.05. The samples are stable and show no signs of aggregation with the low salt ion concentration. No settling of the pm fragments is observed during the course of the experiments. The Triton X-100 (Rohm and Haas, Philadelphia, PA) is used as is. The percentages of Triton are measured as a volume to volume ratio.

The measuring system is described in detail in previous publications (Czégé and Reinisch, 1990, 1991a-c). The scattering is measured as function of the scattering angle from 15° to 60° in 5° increments. The transmission changes along the optical axis are measured simultaneously for the same wavelength range. The transient

scattering signals are normalized with the transient transmission signals. We make the normalization to correct for any changes in the exciting laser intensity when the polarization is changed. Typically, the correction is less than 10%.

In the centrifugation experiments, we used a Microfuge II (Beckman Instruments, Inc., Fullerton, CA) table top centrifuge at the highest speed.

The transient scattering signals are slightly distorted by the transient changes in the transmission of the sample at 320 nm. We have previously corrected this distortion by making measurements as a function of the bR concentration (Czégé and Reinisch, 1990). We have also used a first order approximation to correct the scattering signals (Czégé and Reinisch, 1991b,c). This first order approximation is not necessarily valid in the measurements made here. So, we correct the scattering signals by subtracting 70% of the measured transmission change. The 70% is estimated from measurements on aggregated and gel samples.

The measured scattering transients are described with 25 exponential curves, equally spaced on the log(time) axis (Czégé and Reinisch, 1991c). The choice of exponentials is justified by the exponential nature of the steps in the bR photocycle. This mathematical description causes some smoothing of the data and allows us to make the surface plots using Mathematica (Wolfram Research, Champaign, IL). The curves are then normalized and corrected for transient absorption changes distorting the transient scattering changes. The scattering at each time point as a function of the scattering angle is described with a fourth order polynomial. Again, this allows us to interpolate and make the appropriate contour surface plots with Mathematica.

III. Results

We show contour curves of the intensity of scattered light as a function of time on a logarithmic axis from μs to s and scattering angle from 15° to 60° at pH 4.1. At the top left of Fig. 1 the pm suspension is excited with vertically polarized light (polarized perpendicularly to the scattering plane) at 532 nm. This plot is marked with a "vertical" double arrow. Right at the top of Fig. 1 the pm suspension is excited with horizontally polarized light (polarized parallel to the scattering plane). This plot is marked with a "horizontal" double arrow. The scattering shown in these two plots can be compared to earlier publications where the scattering angle was fixed at 30° (Czégé and Reinisch, 1990; 1991a-c). With the horizontal excitation, a fast initial decrease in the scattering amplitude is observed, followed by a slower increase in the scattering amplitude. The sign of the scattering transients reverses for vertical excitation.

In the bottom half of Fig. 1 we show similar contour curves of the scattering transient amplitudes from pm suspension at pH 4.1 treated with 0.006% Triton X-100. Note that the amplitude of the scattering transients as function of the scattering angle has much more structure after the addition of Triton.

In the top half of Fig. 2 we show contour curves of the scattering transient amplitudes from pm suspensions at pH 8.05. We show both vertically and horizontally polarized excitation light as a function of the log(time) and the scattering angle from 15° to 60°. Again, the scattering at 30° agrees with the transients reported in earlier publications (Czégé and Reinisch, 1991b,c). In the bottom half of Fig. 2 we show the contour curves of the scattering transient amplitudes from the same pm suspensions treated with 0.02% Triton.

We centrifuge the pH 8 pm suspension for 5 minutes. Then we mix the precipitated membranes with the same supernatant and the large scattering transients are attenuated. This procedure can be repeated on the suspension and additional attenuation is observed. In Fig. 3 there are shown the transient scattering and transmissions changes before and after a series of 5 centrifugations. The Triton treatment and the centrifugation procedure stop the transient changes in the membrane curvature. However, the transient absorption changes remain. We did not fully investigate, at this point, how the centrifugation stops the curvature changes. It is shown in Fig. 3 that not only are the scattering kinetics attenuated with centrifugation, but the apparent transmission kinetics have also changed. In Fig. 3 we also compare the normalized differences for both the scattering and absorption transients before and after the centrifugation series. We find that they are the same, within the experimental errors. The noise in the data increases at shorter times due to the nature of the logarithmic averaging.

IV. Discussion

After the Triton treatment, there are significant changes in the pattern of the scattering kinetics. One is that "vertical" and "horizontal" (vertically and horizontally polarized exciting light) scattering kinetics are no longer opposite relative to the transmission transients. They have significantly increased amplitudes (especially at pH 8.05), and they are unidirectional with respect to the excitation polarization over a range of the scattering angle. The other main difference is the oscillation of the scattering components as a function of the scattering angle. The changes in the scattering cross section of the pm fragments during the photocycle

of the bR can be explained by transient changes in the curvature of the membrane fragments (Czégé 1987a,b, 1988; Czégé and Reinisch 1990, 1991b,c). Using this theory of curvature changes, the angular dependence of the scattering kinetics of the Triton treated samples can be understood. We recall that, in a monodisperse sample (theoretically) an oscillation can be observed in the amplitude of the scattering transients as a function of the scattering angle (Czégé 1987b). The oscillations arise from the interference nature of the scattering. The "frequency" of the oscillations depend upon the particle size.

For a constant radius of curvature, the bending angle of the pm depends upon the fragment size. The larger fragments have a greater bending, because the apparent diameter is nonlinear with the bending. It follows that the pm fragments will have a more uniform apparent diameter when the initial curvature increases. We therefore make the plausible assumption that the observed changes in the scattering after the Triton treatment are the result of an increased initial (and transient) bending of the Triton weakened membrane fragments. The idea of the increased initial bending is also in accordance with the scattering transient being independent of the polarization of the exciting light in the Triton treated pm.

Simply note, that the different signs of the scattering transients in the untreated samples are from the photoselection of the polarized exciting flash and that, with the increased bending of the membranes, the photoselection greatly decreases.

To support theoretically the increased bending idea, in Fig.4, we show the calculated amplitudes of the scattered light as a function of the scattering angle for both polarizations of the exciting light. We first consider the pH 8.05 results. The triangular points show the calculated angular dependence of the maximum

scattering amplitude for a small initial curvature. The square points show the calculated angular dependence of the maximum scattering amplitude for a large initial curvature of the pm fragments. The dashed line connects the points for a simulated vertically polarized excitation and the solid line connects the points for a simulated horizontally polarized excitation. The calculations take into account a log normal distribution of membrane fragment sizes with an average diameter of 0.6 μm and a random orientation. The initial curvature is given as $100°/\mu$m for the triangular points and $230°/\mu$m for the square points.

The slowest process at pH 8.05 has been shown to be a decrease in the curvature of the membrane (Czégé and Reinisch, 1991b,c). We therefore calculated the scattering transients for a *reduction* of $80°/\mu$m/excitation factor.* The amount of curvature change depends, of course, on the fraction of bR molecules excited in a membrane fragment. Because of the polarized exciting light and the photoselection, the excitation factor is typically less than 0.4.

The triangular points do, indeed, follow the trends of the slowest scattering process as shown in the top half of Fig. 2. The horizontally polarized excitation leads to a decrease in scattering for the slowest process. This decrease in scattering is small for the small scattering angles and remains negative across the entire range. The vertically polarized excitation shows a slow process with a modest, negative scattering amplitude for the smallest scattering angle. At larger scattering angles, the scattering amplitude becomes positive and increases in size. The calculated

* The excitation factor takes into account the fraction of chromophores properly oriented to absorb an excitation photon. This is not an absolute number. However, the product of the curvature change, the size the excitation factor is an absolute factor. In this case, if the excitation factor is 0.4 for a 0.6 μm membrane fragment, then the curvature reduces 19.2°.

amplitudes vary slightly from the measured amplitudes. This is expected. We used simple square membrane sheets with a uniform cylindrical radius of curvature. The actual situation is probably much more complicated. The close agreement with the sign of the scattering process and the trends in the size of the scattering is remarkably close.

The squares were calculated with an *increase* of curvature of 80°/μm/excitation factor. The change from a decrease to an increase of curvature for the transient process was needed to achieve an agreement between the sign of the measured and calculated scattering amplitudes. The squares agree with slowest process measured in the Triton treated samples at pH 8.05. From the bottom half of Fig. 2, it is clear that the slowest process is positive for small and large scattering angles, and negative for the 30–40° scattering angle range. Also, the vertically polarized excitation gives a larger negative scattering amplitude in this mid range. However, the calculated scattering amplitudes are smaller than the measured scattering amplitudes.

If we increase the transient increase of curvature to 150°/μm/excitation factor (and keep the 230°/μm initial curvature), we calculate scattering amplitudes that are shown as circles on Fig. 4. The amplitudes of the scattering kinetics are increase, but the angular dependence remains. The agreement between the calculations and the measurements is impressive.

A similar analysis of the pH 4.1 is possible. One needs to start with an increase in curvature for the sample without the Triton treatment. The calculated scattering amplitudes are then very nearly the negative of the triangles shown on Fig. 4. After the Triton treatment the curvature still increased, but it is hard to say if the

amount of transient increase changes.

We do not have an explanation why the Triton changes the direction of bending for the pH 8.05 sample. We can assume that only process B in enhanced by the Triton treatment. The altered membranes may not have the posibility to complete process C (Czégé and Reinisch, 1991b,c). We do note that the bending processes also change in time after Triton is added. The analysis is complicated because the Triton not only changes the physical mechanics of the membrane, but also affects the release of non proton ions (see below).

As for the Triton concentrations, at both pH's we use the concentrations at which the scattering changes are maximal. At these concentrations the protein is still not solubilized (cf. centrifugation experiment above). The difference of the necessary concentrations for pH 4.1 and 8.05 indicates that the properties of the membrane changes significantly with the pH. If we follow the effect of adding more Triton at pH 8.05 we find that the scattering changes soon disappear together with the initial scattering intensity. Yet, several hours later, the initial scattering of the solubilized sample is restored. However, the suspension still does not show any transient scattering changes during the photocycle.

It is interesting to note that at pH 8.05 the 0.02% is the same Triton concentration where enhanced nonproton ion movements can be observed during the photocycle (Marinetti and Mauzerall, 1986). We do not have the possibility to measure the transient conductivity of our samples. However, measuring the transient conductivity together with the centrifugation experiment could teach us a lot about the connection of the transient membrane bending and the nonproton ion release.

The distortion of the transient absorption (transmission) changes as shown in Fig. 3 (compare before and after centrifugations) more than likely is due to the effect of the scattering changes. Because of the decreased photoselection, the integrated scattering changes become greatly enhanced which results in a severely distorted transient transmittance of the sample. Even without Triton, the transient scattering changes may significantly distort the transient absorption changes. This is obviously an effect that must be considered in a careful analysis of the absorbance kinetics.

The membrane bending is significant in the study of bR. Measurement of the bending before and after treatment with Triton X-100 shows significant changes in the angular dependence of the scattering transients. Calculations using our model of a bent membrane fragment changing curvature during the photocycle describe the changes well. They suggest that Triton weakens the membrane, leading to an increase in the initial bending angle and an increase in the amount of transient bending also supports our model. We have shown in earlier papers that two of the fundamental bending processes have been associated with the protein conformational changes during the proton pumping process (Czégé and Reinisch, 1990). A third fundamental bending processes has been circumstantially linked to a change of the ion concentrations near the membrane (Czégé and Reinisch, 1991c). Also, the transient scattering amplitude is very sensitive to low levels of photodestruction (Czégé and Reinisch, 1991a). Thus, the transient scattering is sensitive to critical aspects of bR, where the absorbance measurements do not show such sensitivity. In addition, changes in the scattering cross section can and will influence the changes in absorption of the pm observed during the photocycle. Understanding

the changes in the scattering is important in the understanding of the mechanism of the proton pump.

Acknowledgements

We thank J.K. Lanyi and György Váró for the generous supply of pm used in these experiments. This work has been supported in part by grants from the Office of Naval Research, Grant N00014- WR-24020 and the Strategic Defense Initiative Organization under the Medical Free Electron Laser program. The opinions and assertions contained in this paper are the private views of the authors and are not to be construed as reflecting the views of the Uniformed Services University of the Health Sciences or the Department of Defense.

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Figure Captions

Figure 1: Contour plots of the scattering of light at 320 nm from suspensions of pm during the photocycle. The suspensions are approximately 5 μM bR and at pH 4.1. The exciting light is at 532 nm (20 ns laser flash). The horizontal axis is the logarithmic time during the photocycle from μs to s. The vertical axis is the scattering angle, linear from 15° to 60°. Each contour line is a 0.3% change in the intensity of the scattered light. The shading shows the sign of the scattering transients. Lightly shaded regions have positive changes (increases) in scattering and dark regions have negative changes (decreases) in scattering. The shading near 1 s represents a zero percent change in scattering. The data was described with 25 exponential curves, and the absorption changes have been subtracted from the data shown as explained in the text. The top half of the figure is from pm not treated with Triton. The vertical double arrow designates the data from vertically polarized excitation. The horizontal double arrow designates the data from horizontally polarized excitation. The bottom half of the figure is from pm treated with 0.006% Triton X-100. In the bottom half of the figure each contour line is a 0.1% change in the intensity of the scattered light.

Figure 2: The same as Fig. 1, except the sample is at pH 8.05. In the top half of the figure, without the Triton treatment, each contour line is a 0.1% change in the intensity of the scattered light. In the bottom half of the figure, the Triton treatment is 0.02%, and each contour line is a 0.3% change in the intensity of the scattered light.

Figure 3: The scattering change. The percent change in scattering near 320 nm from a suspension of pm, pH 8.05, treated with 0.02 % Triton on a loga-

rithmic time scale. The 5 μm suspension of bR is excited with 20 ns pulse at 532 nm. The dashed line is from the Triton treated sample. The solid line is from the same sample centrifuged five times and resuspended, as explained in the text. The scattering angle is 20°.

The transmission change. The percent changes in transmission near 320 nm from the same bR suspension. These data were measured simultaneously with the scattering changes. Again, the dashed line is from the Triton treated sample. The solid line is after the same sample was centrifuged and resuspended five times.

The normalized differences. The normalized differences in the scattering and transmission transients before and after the centrifugation. The dashed line is the difference in the scattering transients. The solid line is the difference in the transmission transients.

Figure 4: The calculated amplitudes for the scattering transients at 320 as a function of the scattering angle. The details of the calculation are given in the text. The triangular points are from a 100°/μm initial curvature and a *decrease* in curvature of 80°/μm/excitation factor. The square points are from a 230°/μm initial curvature and an *increase* in curvature of 80°/μm/excitation factor. The circles are from a 230°/μm initial curvature and an *increase* in curvature of 150°/μm/excitation factor.

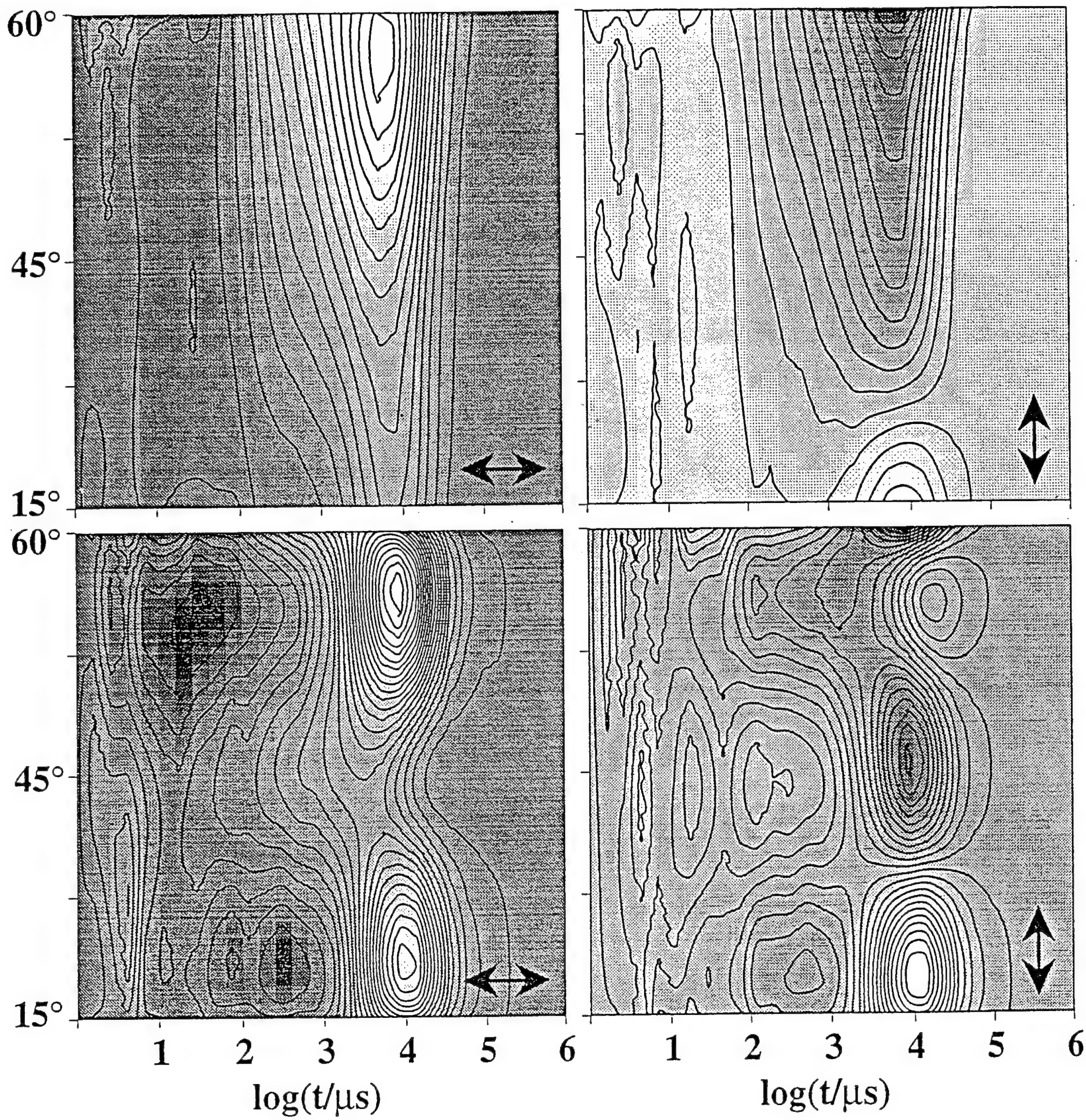

Figure 1

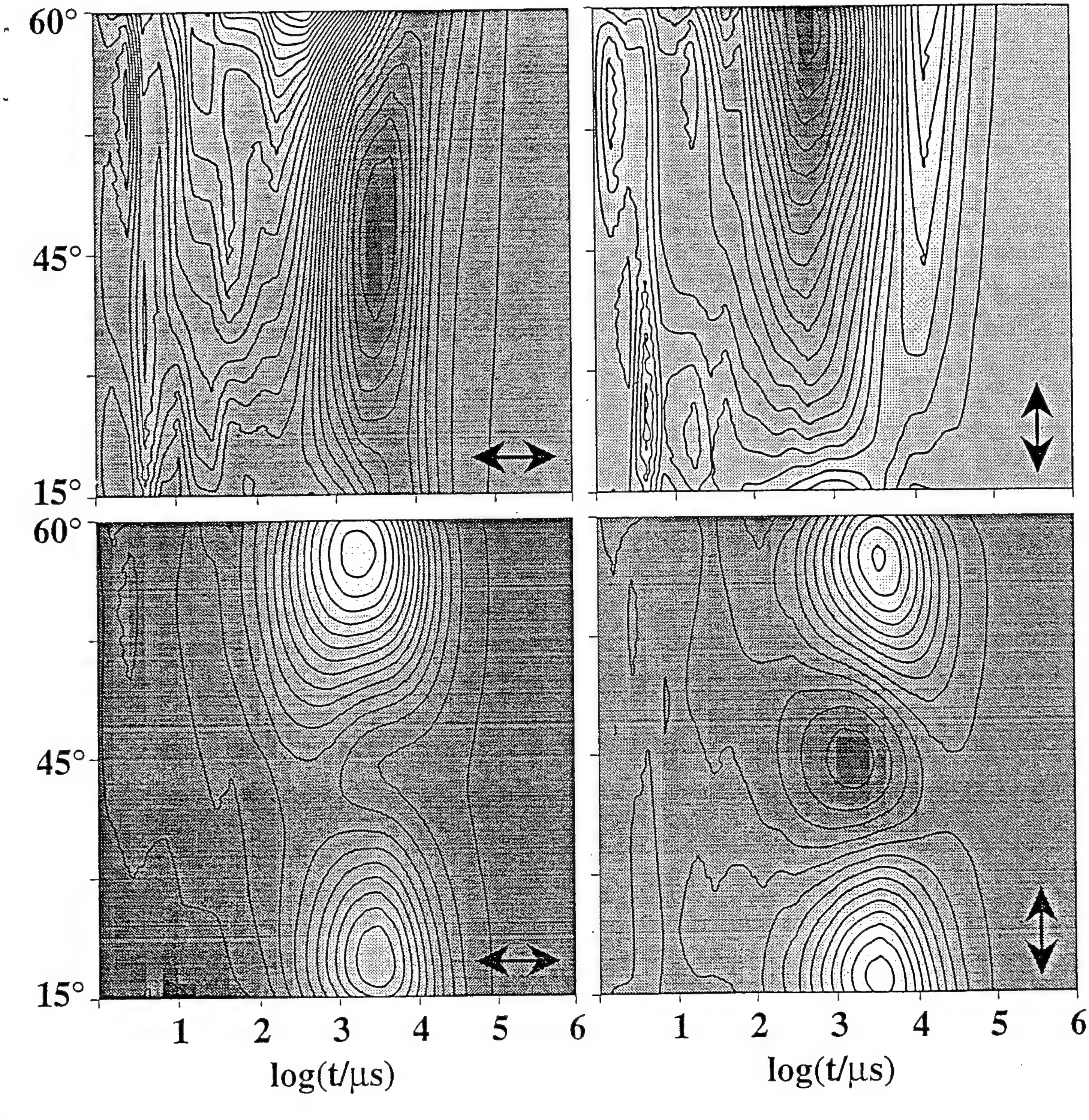

Figure 2

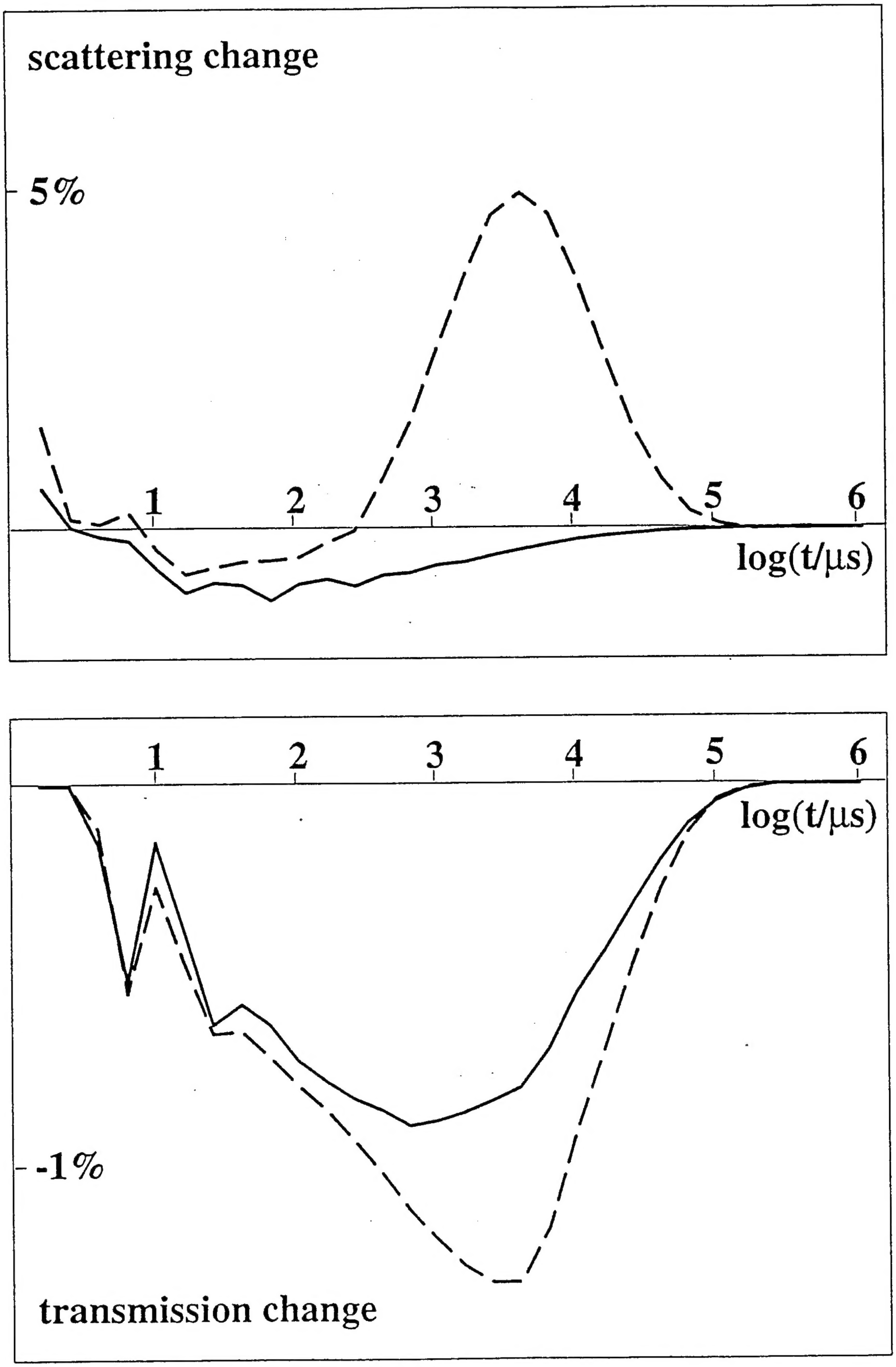

Figure 3 (page 1 of 2)

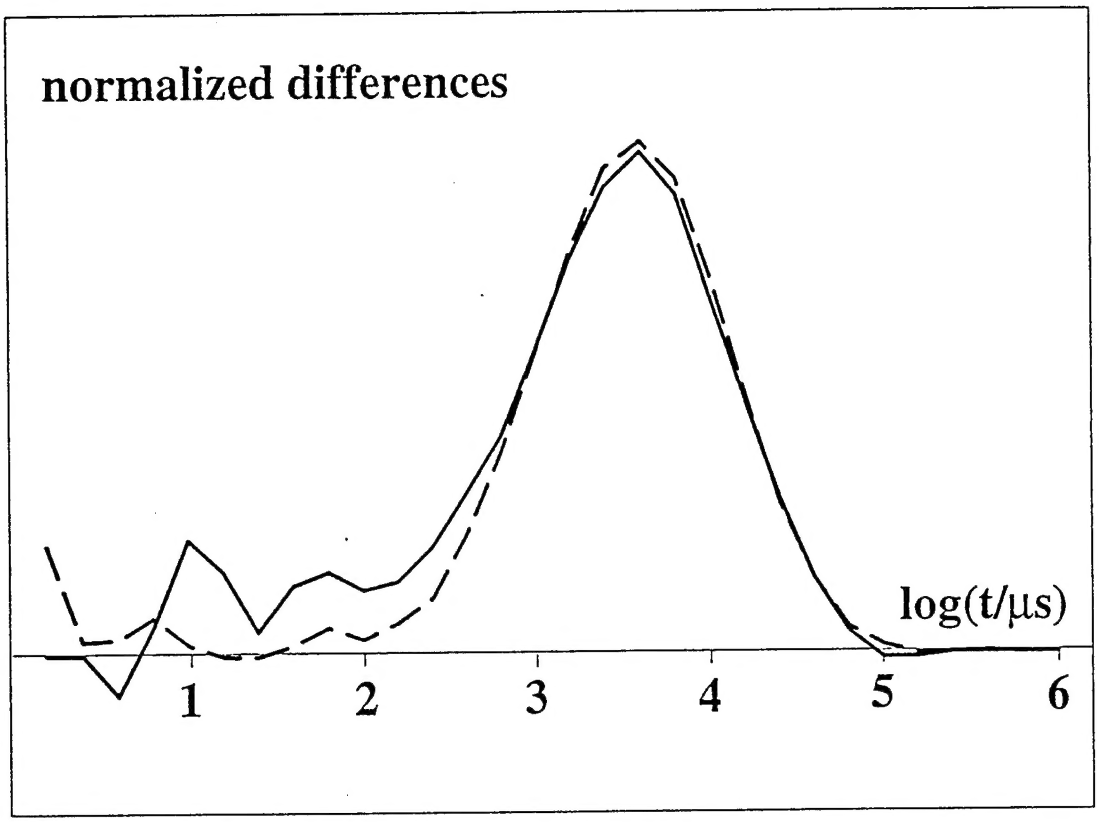

Figure 3 (page 2 of 2)

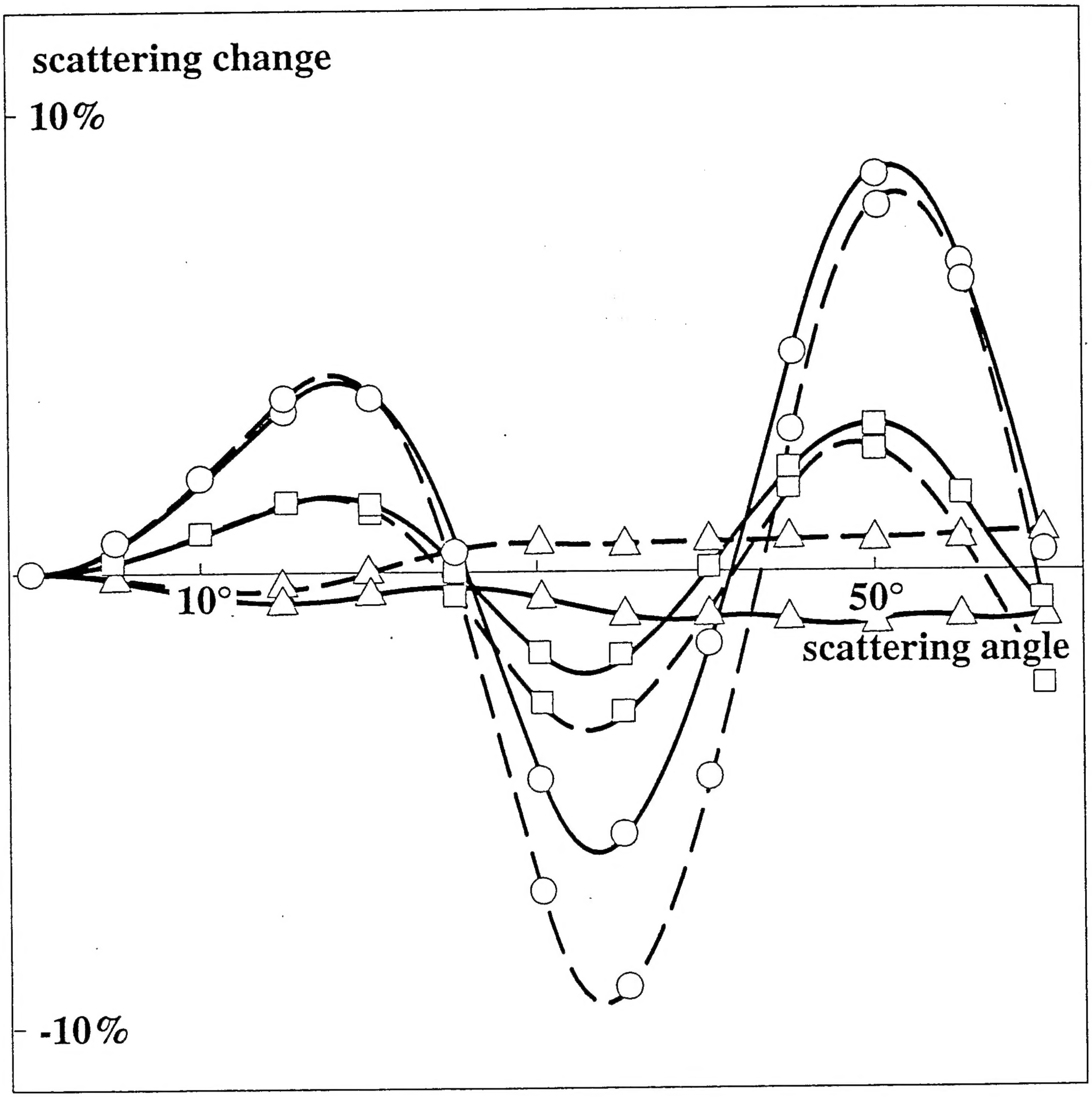

Figure 4

Research supported by Vanderbilt FEL from L Reinisch
10 January, 1992

1. Fluorescence detection and identification in the management of acute otitis media.

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Fluorescence Spectroscopy of Bacteria in Otitis Media, J.A. Tribble, J. Werkhaven and L. Reinisch, Applied Spectroscopy (in preparation).

Abstract

The management of otitis media in children continues to be hampered by two fundamental problems: The prevalence of bactericidal resistant species is increasing (frequent misdiagnosis lead only to enhancement of the growth conditions for resistant species); The identification of resistant species is time consuming, expensive and requires drainage of the middle ear. The fluorescence spectra of four strains of bacteria, commonly found in otitis media: *P. aeruginosa, S. aureus, B. catarrhalis* and *H. influenzae* has been measured. The excitation wavelength has been varied from 280 to 500 nm and the emission spectra measured. The fluorescence spectra are presented at two dimensional fluorescence finger prints. These fingerprints will ultimately be used to identify the bacteria remotely, and non invasively from otitis media.

Future Research Support:
(i) An Innovative Technology Research Grant to the National Institutes of Health is being prepared and will be submitted February 1, 1992.

(ii) The Deafness Research Foundation has been contacted. A proposal will be submitted before their annual due date in June, 1992.

(iii) We are in the initial stage of contacting the Whitaker Foundation for support.

2. Measurement of the membrane dynamics of the activated purple membrane.

Collaborator: J. Czégé
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The Effect of Triton X-100 on Purple Membrane as Measured by Changes in the Dynamics. J. Czégé and L. Reinisch, Photochem. and Photobiol (submitted, 1992).